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The PDZ-binding motif of the avian NS1 protein affects transmission of the 2009 influenza A(H1N1) virus

Jin Il Kim [a,b,1], Min-Woong Hwang [a,b,1], Ilseob Lee [a,b,1], Sehee Park [a,b,1], Sangmoo Lee [a,b], Joon-Yong Bae [a,b], Jun Heo [b], Donghwan Kim [b], Seok-Il Jang [b], Mee Sook Park [a,b], Hyung-Joo Kwon [b], Jin-Won Song [a], Man-Seong Park [a,b,*]

[a] Department of Microbiology, College of Medicine, and the Institute for Viral Diseases, Korea University, Seoul 136-705, Republic of Korea
[b] Department of Microbiology, College of Medicine, Hallym University, Chuncheon, Gangwon-do 200-702, Republic of Korea

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ABSTRACT

By nature of their segmented RNA genome, influenza A viruses (IAVs) have the potential to generate variants through a reassortment process. The influenza nonstructural (NS) gene is critical for a virus to counteract the antiviral responses of the host. Therefore, a newly acquired NS segment potentially determines the replication efficiency of the reassortant virus in a range of different hosts. In addition, the C-terminal PDZ-binding motif (PBM) has been suggested as a pathogenic determinant of IAVs. To gauge the pandemic potential from human and avian IAV reassortment, we assessed the replication properties of NS-reassorted viruses in cultured cells and in the lungs of mice and determined their transmissibility in guinea pigs. Compared with the recombinant A/Korea/01/2009 virus (rK09; 2009 pandemic H1N1 strain), the rK09/VN:NS virus, in which the NS gene was adopted from the A/Vietnam/1203/2004 virus (a human isolate of the highly pathogenic avian influenza H5N1 virus strains), exhibited attenuated virulence and reduced transmissibility. However, the rK09/VN:NS-PBM virus, harboring the PBM in the C-terminus of the NS1 protein, recovered the attenuated virulence of the rK09/VN:NS virus. In a guinea pig model, the rK09/VN:NS-PBM virus showed even greater transmission efficiency than the rK/09 virus. These results suggest that the PBM in the NS1 protein may determine viral persistence in the human and avian IAV interface.

1. Introduction

Influenza A viruses (IAVs) have a genome consisting of 8 segmented, negative-sense and single-stranded RNAs, which encode 8 major structural proteins and two nonstructural (NS) proteins as well as other minor proteins [1,2]. Due to the nature of their genome, IAVs have the potential to generate variants through a reassortment process, which is a swapping of gene segments in a cell co-infected with two different strains of IAVs [3,4]. Genetic reassortment played a critical role in the appearance of pandemic IAVs in 1957, 1968, and 2009 [5].

The NS gene is the shortest segment of the IAV genome and encodes the NS1 and NS2 (or nuclear export protein, NEP) proteins, of which the latter is produced from a spliced form of the NS mRNA [1]. The NS1 protein is multifunctional and mainly involved in counteracting the host's innate immune responses via the inhibition of type I interferon (IFN) production. NS1 has been known also to interact with a variety of host proteins [6], to evolve in a host lineage-dependent manner [7] and to be directly involved in viral RNA replication and protein synthesis [8]. The growth of IAVs with truncated NS1 has been shown to be severely compromised in IFN-response competent cells [9], and these IAVs produced high pathogenicity only in the IFN signaling-deficient mice [10]. The NS2 protein is required for the export of a stoichiometrically composed ribonucleoprotein (RNP) complex of viral RNA segments from the nucleus, where influenza virus genome replication occurs, to the cytoplasm for packaging into a new virion [11].

The NS1 and NS2 proteins share an N-terminal 10 amino acid (aa) region, and the mRNA sequence translated for the C-terminus of the NS1 overlaps with part of the translated NS2 mRNA in a different reading frame [12]. Interestingly, the influenza NS1 sequence of different host species displays certain patterns in its length [13]. Two generalized patterns include the deletion of 5 aa between the RNA binding and effector domains in the

* Corresponding author at: Department of Microbiology, College of Medicine, and the Institute for Viral Diseases, Korea University, Seoul 136-705, Republic of Korea. Fax: +82 2 923 3645.
E-mail address: manseong.park@gmail.com (M.-S. Park).
[1] These authors contributed equally to this article.

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H5N1 NS1 proteins [14] and the presence or absence of the PDZ-binding motif (PBM) [13,15]. Studies on the effects of these pattern alterations on viral pathogenicity have suggested that they may be virulence determinants [16,17]; however, contrary examples in nature and in published reports invite further investigation [13,18]. There has been a predominant presence of PBM-truncated NS1 proteins through an earlier stop codon change in the later classical swine influenza H1N1 subtypes, which include the 2009 pandemic H1N1 (pH1N1) virus, although the 1918 pandemic (p1918) and early H1N1 viruses possessed the NS1 with the PBM [13].

The restoration of the PBM in the NS1 protein of the A/California/04/2009 (CA04; pH1N1) virus has been shown to have no significant effect on the viral growth in cultured cells, pathogenicity in mice and transmission in ferrets [19]. In this study, we investigated the effects of the avian-origin NS and the PBM extension in its NS1 protein on the growth and IFN-antagonizing properties of the pH1N1 K09 virus. Pathogenesis and transmission sequelae were then assessed in animal models.

2. Materials and methods

2.1. Cells and viruses

Madin Darby canine kidney (MDCK), swine kidney epithelial (LLC-PK1), human lung epithelial-(A549), and African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in media supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco, Carlsbad, CA) in an incubator at 37 °C and 5% CO_2.

Full gene plasmids of the A/Korea/01/2009 (K09; 2009 pandemic H1N1 strain) virus were constructed into a bidirectional pDZ plasmid. The PBM was absent in the K09 NS (Fig. 1A). In the pDZ/VN:NS (kindly provided by Dr. Peter Palese at the Icahn School of Medicine at Mount Sinai, New York, NY) of the A/Vietnam/1203/2004 (VN; the human isolate of a highly pathogenic avian influenza H5N1 virus), a T646A nucleotide substitution was introduced to generate the pDZ/VN:NS-PBM plasmid, which would express the C-terminal E-S-E-V-like PBM-extended VN:NS1 protein without affecting the VN:NS2 open reading frame (ORF) (Fig. 1B and C). The rK09, rK09/VN:NS, and rK09/VN:NS-PBM viruses were then generated by reverse genetics using the corresponding NS plasmid in the K09 virus backbone [20]. The rescue efficiency was comparable between viruses (based on the plaque titers in the initial purification step). The recombinant Newcastle disease virus expressing green fluorescent protein (rNDV-GFP; provided by Dr. Peter Palese) was used to evaluate the interferon (IFN)-antagonizing activity of the rescued viruses. All of the viruses were propagated in fertile chicken eggs and purified by a standard plaque assay in MDCK cells for the viral stock preparation. The stock viruses were confirmed by reverse transcriptase-PCR and subsequent sequence analysis before use.

2.2. Growth kinetics analysis of the viruses in cell lines

Multiple- (multiplicity of infection of 0.01, MOI = 0.01) and single-cycle (MOI = 2) growth kinetics of the viruses were performed in MDCK, LLC-PK1, and A549 cells. Briefly, the cells were infected with each virus for 1 h and rigorously washed with PBS. The infected cells were then maintained with virus growth media in an incubator at 37 °C and 5% CO_2. Cell supernatants were collected at the indicated time intervals and used for virus titration by plaque assay in MDCK cells.

2.3. IFN inhibition efficiency of the viruses using the rNDV-GFP virus

To evaluate the IFN-antagonizing activity of the viruses [21], A549 cells were inoculated with the rK09, rK09/VN:NS, or rK09/VN:NS-PBM virus at an MOI of 1. The cell supernatants were collected at 24 h post-infection (hpi) and treated with UV light for 15 min on ice. The absence of infectious viral particles in the UV-treated cell supernatants was confirmed by plaque assays in MDCK cells. Vero cells, which lacked the IFN-antagonizing effects, were then treated with the cell supernatants for 16 h and subsequently inoculated with rNDV-GFP (MOI = 1). At 24 hpi, GFP expression was determined using a fluorescence microscope (EVOS, Advanced Microscopy Group, Bothell, WA). Recombinant human IFN-β (250–1,000 U/ml; PeproTech, Rocky Hill, NJ) was used as a control.

2.4. IRF-3 nuclear translocation analysis using confocal microscopy

To evaluate the inhibitory efficiency of the NS1 protein against IRF-3 nuclear translocation, the pCAGGSII expression plasmid of the IRF-3/GFP fusion protein (pIRF-3/GFP; provided by Dr. Peter Palese) was transfected into A549 cells together with each NS1 expression plasmid (pK09:NS1, pVN:NS1 and pVN:NS1-PBM) for 24 h. The cells were then infected with a Sendai virus (SeV), which lacked IFN-antagonizing effects, at an MOI of 1 and kept under standard cell culture conditions. At 16 hpi, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed with cold PBS. The cell nuclei were then stained with Hoechst 33258 (Sigma, St. Louis, MO), and IRF-3 nuclear translocation was determined by the GFP localization, as observed using a confocal microscope.

2.5. Animal experiments

2.5.1. Ethics statement

This study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Animal, Plant, and Fisheries Quarantine and Inspection Agency of Korea, and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Hallym University (permit number: Hallym 2010-99, 2013-48).

2.5.2. Mouse experiments

To study viral pathogenesis in mice, five BALB/c mice (5–6 weeks old, female; NARA Biotech, Seoul, Korea) were intranasally infected with 10^6 plaque forming units (PFU) of each virus, and body weight changes were monitored daily for 14 days post infection (dpi). Body weight changes were determined by comparing the body weight of each mouse to its initial weight. Viral replication in the lungs was also determined by sacrificing three mice at 3 and 6 dpi, respectively.

2.5.3. Viral transmission in a guinea pig model

Virus transmission was evaluated in guinea pigs. After intranasal infection (10^5 PFU) with each virus, an infected guinea pig was co-housed with a naïve guinea pig the next day. To determine viral transmission, nasal washing was performed using PBS supplemented with penicillin–streptomycin and 0.3% bovine serum albumin onto a sterilized Petri dish every two days starting from 1 dpi or one day post-exposure (dpe). After the removal of nasal debris by centrifugation, the nasal wash samples were used for viral titration.

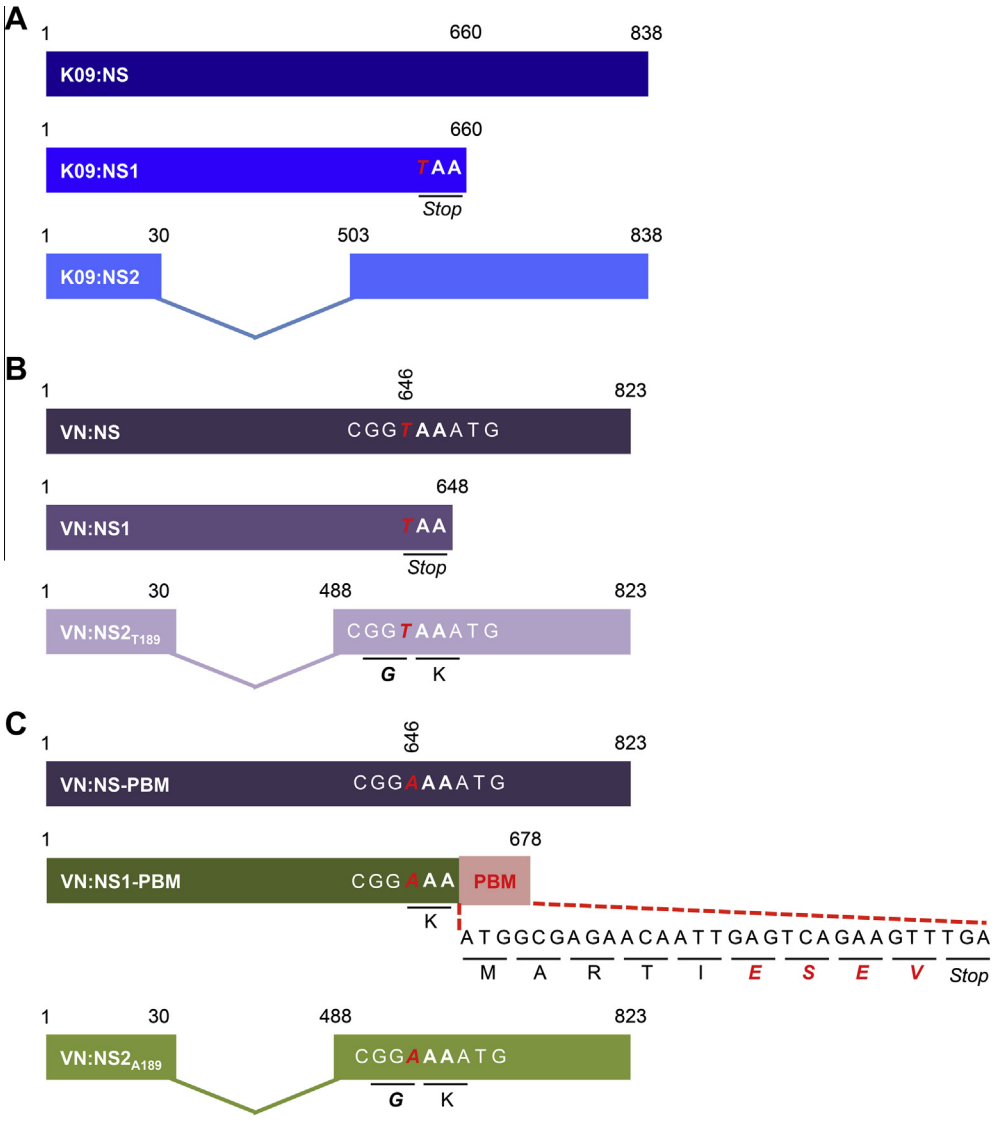

Fig. 1. Schematic representations of the different NS gene expressions. (A) Using an alternative splicing method, the K09 NS gene produces two proteins, NS1 (from 1 to 660 in nts) and NS2 (1–30 then 503–838; 366 nts), in the virus-infected cells. (B) The VN:NS gene also expresses two proteins (NS1, 1–648; NS2, 1–30 then 488–823). (C) Due to the T646A (T189A in the NS2 nts number) nucleotide substitution, the C-terminus of the VN:NS1 protein extends to 678 nts (30 nts extension) and retains the PDZ-binding motif (PBM) without changing the NS2 ORF. Stop refers to stop codons (TAA and TGA). G, glycine; K, lysine; M, methionine; A, alanine; R, arginine; T, threonine; I, isoleucine; E, glutamic acid; S, serine; and V, valine.

3. Results and discussion

3.1. Generation of the NS-reassorted viruses

The NS gene evolution rate appeared to be dynamic rather than conserved, similar to that of hemagglutinin, which undergoes continuous antigenic drift [22]. Sequence variation at the C-terminal end of NS1 has been shown to be especially dynamic [13]. Obenauer et al. identified a potential PBM (X-S/T-X-V) in this region that would interact with the PDZ domain-containing proteins in hosts and hypothesized that the PBM might function as a virulence determinant [15]. Subsequently, it was shown that PBM substitution in the A/WSN/33 (WSN) virus NS1 with the PBM of avian types increased viral pathogenesis in mice. The truncation of this motif greatly attenuated viral replications in both cultured cells and in mice [17]. However, recovery of the PBM in the CA04 NS1 protein neither increased virulence nor enhanced viral transmissibility [19].

The NS1 protein of the H5N1 VN virus also lacks the PBM (Fig. 1B), although this motif could be restored by a T646A nucleotide substitution, which would extend the NS1 ORF to 678 nucleotides (nts) without affecting the NS2 ORF (Fig. 1C). The natural appearance of such a change in a pH1N1 isolate [19] prompted us to consider the possibility that an NS gene of avian origin might get reassorted with other human IAV gene segments and acquire the earlier stop-abolishing mutation. Such an event may potentially occur in the context of an H5N1 outbreak in humans [23]. Therefore, to determine whether the NS1 PBM affects the pathogenesis and transmission efficiency of the human and avian IAV reassortants, the NS-reassorted viruses rK09/VN:NS and rK09/VN:NS-PBM were generated.

3.2. Growth properties of the NS-reassorted viruses in cultured cells

The growth properties of the rK09, NS-reassorted rK09/VN:NS and rK09/VN:NS-PBM viruses were analyzed in MDCK, LLC-PK1, and A549 cells. In MDCK cells, all of the viruses replicated similarly, reaching $\sim 10^8$ PFU at 24 hpi (Fig. 2A). Only the rK09/VN:NS strain was attenuated in the single-cycle replication kinetics (Fig. 2D). MDCK cells are permissive to various IAVs, partially due to a lack

of anti-influenza activity of canine Mx proteins [24]. Therefore, growth profiles in MDCK cells may serve as a positive control [25].

Because human and swine IAVs could infect the reciprocal hosts [26] and reassorted variants between them might arise in pigs [27], we subsequently determined growth profiles of the viruses in LLC-PK1 cells. In these cells, the rK09/VN:NS-PBM virus outgrew the other two viruses between 16 and 24 hpi, regardless of the replication cycle settings (Fig. 2B and E). Interestingly, both NS-reassorted viruses were highly attenuated in human A549 cells as compared with the replication of the rK09 virus, which might have resulted from a lack of gene compatibility or species-specific NS activity (Fig. 2C and F). However, similar to the results in LLC-PK1 cells, the rK09/VN:NS-PBM virus exhibited a higher replication efficiency than the rK09/VN:NS virus in A549 cells. It was apparent that the avian NS-mediated growth defects were compensated in the replication kinetics by the PBM extension in the same NS gene. These results suggest that avian NS-mediated viral incompetent growth in a different host may be offset by the PBM extension.

3.3. IFN-antagonizing activity of the NS-reassorted viruses

We determined whether the growth profile of the aforementioned viruses is correlated with the IFN-counteracting activity of the viruses. We used an rNDV-GFP-based assay system to measure IFN induction rates in the cells after infection with NS-reassorted viruses. Due to the high sensitivity of the rNDV-GFP virus to IFNs, expression of the NDV genes (thus also expression of GFP) could be inhibited in a cell pretreated with IFN [28]. We pretreated IFN production-deficient Vero cells [29] for 16 h with supernatants of human A549 cells that had been infected with rK09, rK09/VN:NS, or rK09/VN:NS-PBM virus and subsequently infected the Vero cells with rNDV-GFP. The results showed that the rK09 virus was more efficient in inhibiting IFN production in A549 cells than the rK09/VN:NS or rK09/VN:NS-PBM virus, and a higher IFN inhibitory efficacy was observed with the rK09/VN:NS-PBM virus than with the rK09/VN:NS virus (Fig. 3A). Although there might be some discrepancy between avian IAV replication in human cells and virus-induced IFN production [30], the IFN production profiles

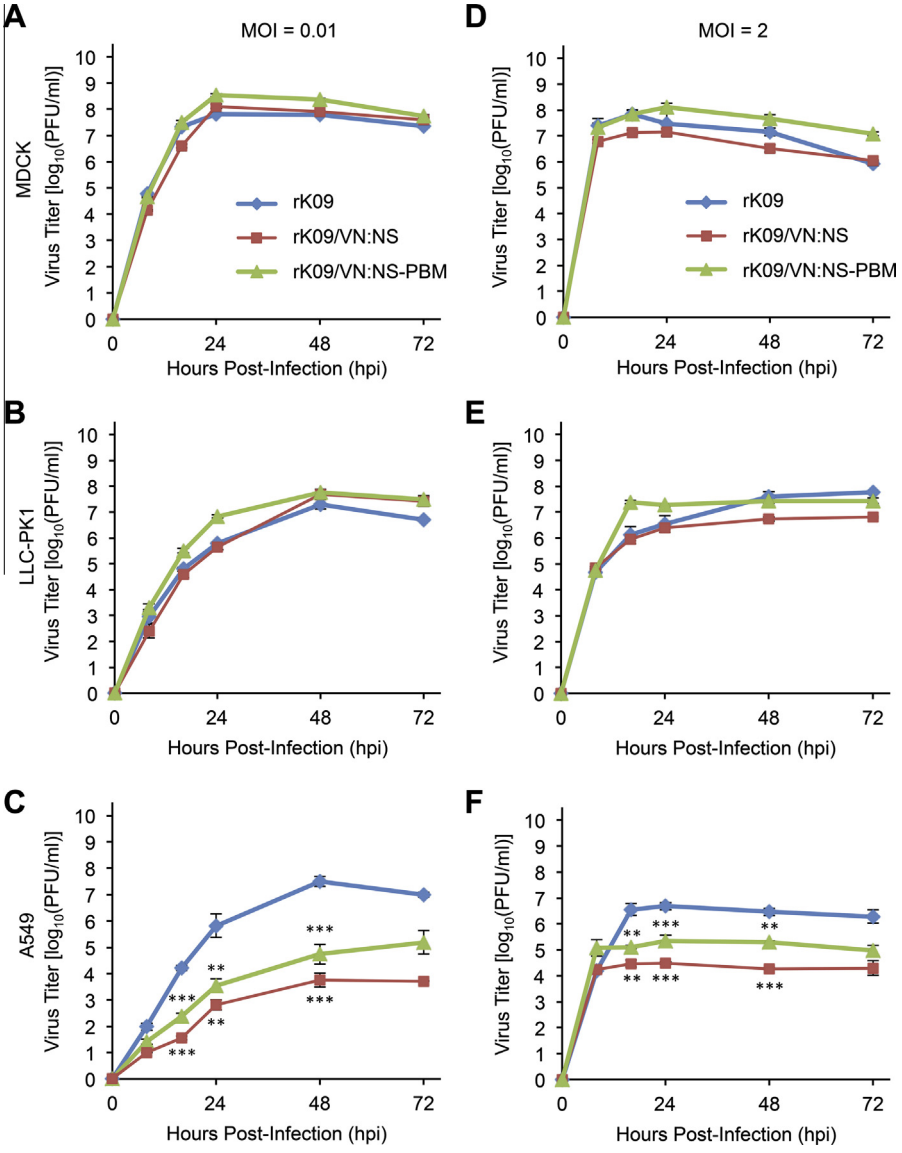

Fig. 2. Growth characteristics of the viruses in different cell lines. MDCK (A and D), LLC-PK1 (B and E), and A549 (C and F) cells were inoculated with the rK09, rK09/VN:NS, or rK09/VN:NS-PBM virus for multi- (MOI = 0.01 in A, B, and C) or single-cycle (MOI = 2 in D, E, and F) replication kinetics analyses. Results represent the average values of triplicate analyses. Error bars denote the standard deviation (SD). The statistical significance of differences in the viral growth rate compared with that of the rK/09 virus was assessed by a two-tailed, unpaired Student's *t* test (**, $P < 0.01$; ***, $P < 0.001$).

were commensurable to the growth kinetics of the tested viruses in A549 cells (Fig. 2C and F).

The NS1 protein of IAVs has been known to inhibit IRF-3 activation by blocking its translocation from the cytoplasm to the nucleus, and transfected NS1 proteins could function *in trans* in the same manner [31]. After transfecting expression plasmids of the K09:NS1, VN:NS1, or VN:NS1-PBM protein, we examined the inhibition of the IRF-3 nuclear translocation in A549 cells. In this assay, the IFN-antagonizing activity of different NS1 proteins was clearly demonstrated by detecting (Fig. 3B) and quantifying (Fig. 3C) the GFP signals. The IRF-3 nuclear translocation rate of the pVN:NS1-transfected cells was similar to that of the mock (empty vector)-transfected cells, indicating that the VN:NS1 protein was incompetent against IFN production and explaining why the rK09/VN:NS virus was severely attenuated in A549 cells (Fig. 2C and F). However, the nuclear translocation of IRF-3 was blocked by 27.96% by the VN:NS1-PBM, although at a lower level than in pK09:NS1-transfected cells (45.73%) (Fig. 3B and C). The possibility of different transfection efficiencies of the NS1 plasmids was excluded based on the comparable rescue efficiency of the corresponding viruses. These results support the hypothesis that the PBM extension compensates for the viral incompetency of the avian NS-reassorted human IAV variant.

3.4. Pathogenesis and transmission efficiency of the NS-reassorted viruses in animal models

We next assessed the pathogenicity of the viruses in mice. When considering the body weight changes of the infected mice, the rK09/VN:NS virus (2.53 ± 3.29% weight loss at 6 dpi) was less pathogenic than rK09/VN:NS-PBM (14.94 ± 3.17% at 6 dpi), which produced a similar weight loss as the rK09 strain (14.63 ± 8.57% at 6 dpi) (Fig. 4A). Viral replication in the lungs also revealed the lower pathogenicity of the rK09/VN:NS virus ($10^{6.10 \pm 0.01}$ and

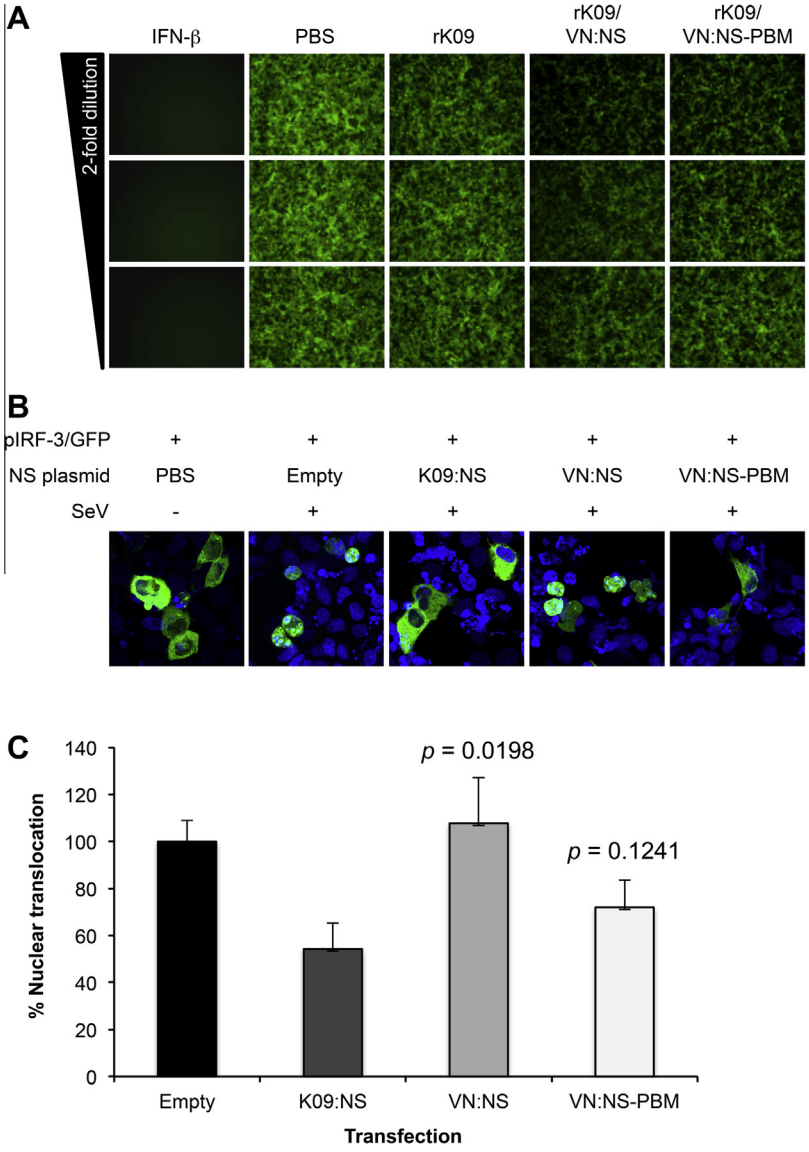

Fig. 3. Interferon-antagonizing activity of the viruses in A549 cells. (A) Different amounts of induced IFN in A549 cells by the infection of rK09, rK09/VN:NS, or rK09/VN:NS-PBM virus repressed rNDV-GFP viral replication in Vero cells. GFP expression was determined using a fluorescence microscope. Recombinant human IFN-β (250–1,000 U/ml) and PBS were used as controls. (B) Using confocal microscopy, IRF-3/GFP nuclear translocation was determined by transfection together with the K09:NS1, VN:NS1, or VN:NS1-PBM expression plasmid and subsequent SeV infection. An empty vector and PBS were used as controls. (C) The number of cells with nuclear GFP signal relative to the total number of GFP-expressing cells in each condition was plotted as a percentage. Results represent the average values of triplicate analyses. Errors bar denote the SD. The statistical significance of differences in the inhibition of IRF-3/GFP nuclear translocation compared with that of K09:NS1 expression was assessed using a two-tailed, unpaired Student's *t* test.

$10^{6.84 \pm 0.09}$ PFU at 3 and 6 dpi, respectively), which might be associated with the virus titer in the lungs at 3 dpi (Fig. 4B). The attenuated virulence of the rK09/VN:NS virus in A549 cells was similarly reflected in its pathogenicity in mice. However, this attenuation was recovered by the PBM-extended rK09/VN:NS-PBM virus ($10^{6.70 \pm 0.09}$ and $10^{6.71 \pm 0.12}$ PFU at 3 and 6 dpi, respectively), which persisted at a higher level than rK09 ($10^{6.90 \pm 0.09}$ and $10^{6.38 \pm 0.26}$ PFU at 3 and 6 dpi, respectively) until 6 dpi (Fig. 4B).

Subsequently, we sought to assess the viral transmissibility. We previously reported guinea pigs as a relevant animal model for studying the direct transmission of the pH1N1 K09 virus [32]. Therefore, we determined the transmission profiles of the rK09, rK09/VN:NS, and rK09/VN:NS-PBM viruses using the same guinea pig model (Fig. 4C–F). The results showed that the rK09/VN:NS-PBM virus replicated at comparable peaks in the upper respiratory tract of guinea pigs and was transmitted to exposed guinea pigs at a higher efficiency (10/12, 83.3%) than was the rK09 strain (4/6, 66.7%) (Fig. 4D and F); in contrast, rK09/VN:NS showed a low level of transmission and the replication peaks (4/12, 33.3%) (Fig. 4E). Taken together, these results suggest that the avian NS gene functions as a deleterious component of the human and avian IAV reassortment variant, and the PBM extension may compensate for the viral incompetent transmission of avian NS-harboring variants.

In this study, we simulated the pathogenicity and transmissibility of possible human and avian IAV offspring with an emphasis on the NS-reassorted variants and E-S-E-V-like NS1 PBM extension [17]. Consistent with other studies, the PBM extension functioned as a pathogenic factor in mice. In addition, we determined that the transmissibility of the avian NS-harboring virus can be enhanced by the PBM extension. This study suggests that the NS1 PBM can

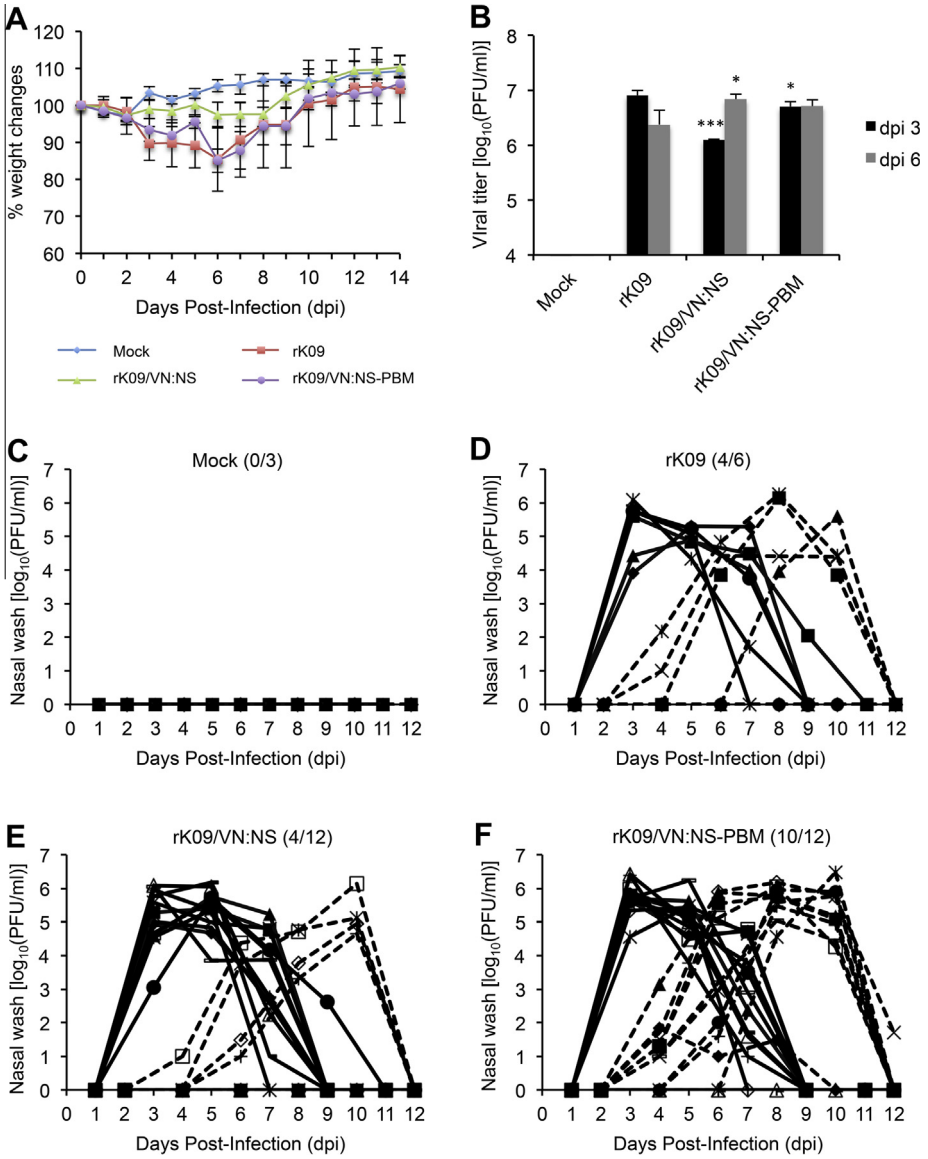

Fig. 4. Pathogenesis and transmission efficiency of the viruses in animal models. (A and B) BALB/c mice were infected intranasally with 10^6 PFU of rK09, rK09/VN:NS, or rK09/VN/NS-PBM virus. (A) Body weight changes were monitored daily until 14 dpi. (B) Virus replication in the lungs was assessed at 3 and 6 dpi by sacrificing three mice from each virus trial. PBS-infected mice (mock group) were used as a control. Error bars denote the SD. The statistical significance of the differences in the virus titer in the lungs compared with that of the rK/09 virus was assessed using two-tailed, unpaired Student's *t* test (*, $P < 0.05$; ***, $P < 0.001$). (C–F) The transmission efficiency of the viruses was assessed in a guinea pig model. Six (D, rK09) or twelve (E, rK09/VN:NS; F, rK09/VN:NS-PBM) infection/exposure guinea pig sets were used for the direct contact transmission analyses. PBS-infected guinea pigs (C, mock group) were used as a control. Solid lines represent infected guinea pigs, and dashed lines represent exposed animals. Each symbol represents an individual guinea pig. The transmission efficiency of the viruses is presented as a ratio (number of transmitted guinea pigs relative to the total number of exposed animals) in parenthesis.

be a transmission determinant of IAVs in the sporadic but persistent human infections by avian H5N1 viruses.

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